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Critical Analytical Testing Considerations in the Era of Citrus Greening

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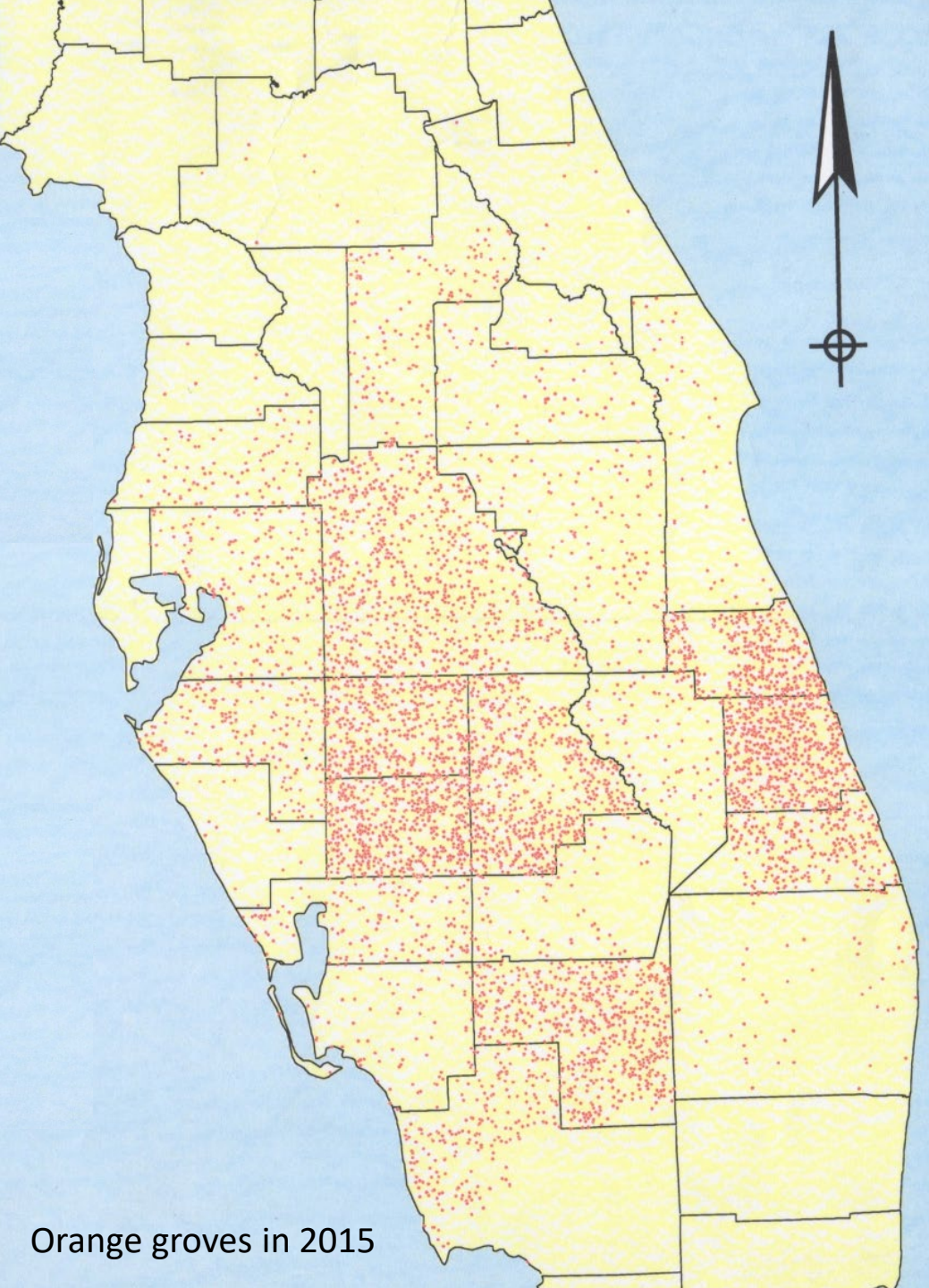
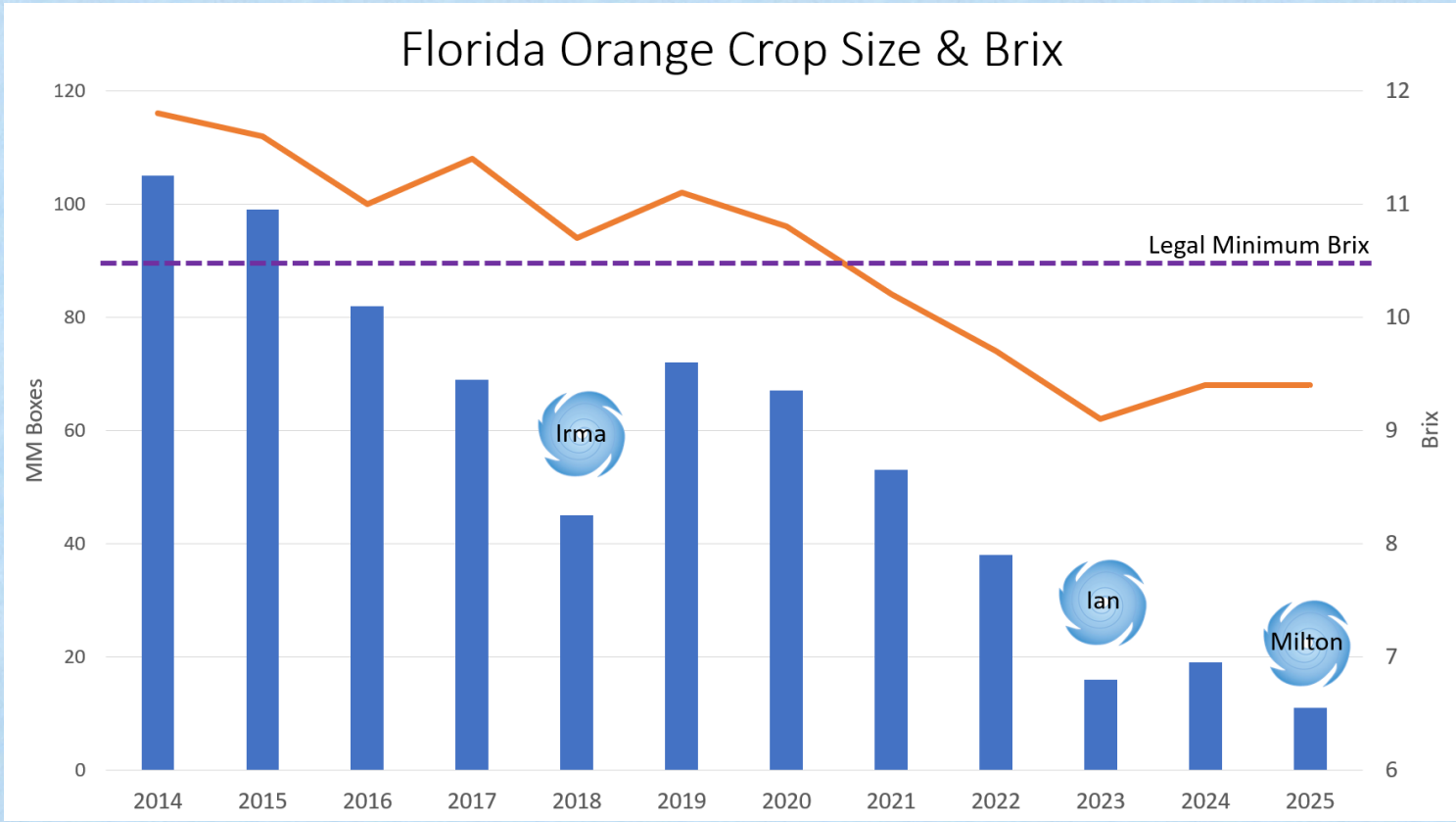
The Coca-Cola Company

FLORIDA



Orange groves in 2015



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Key Measurements for Liking

- Brix
- Acid
- Color
- Taste → Limonin

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Limonin Testing

LC-UV

Usually involves SPE, nomilin not quantifiable

LC-MS

ESI APCI

LC-MS/MS

ESI APCI

Dilute and Shoot- Nomilin Detectable

Increasing Cost, Instrumental Complexity, Sample Prep Ease

Decreasing Sensitivity, Specificity, Cost

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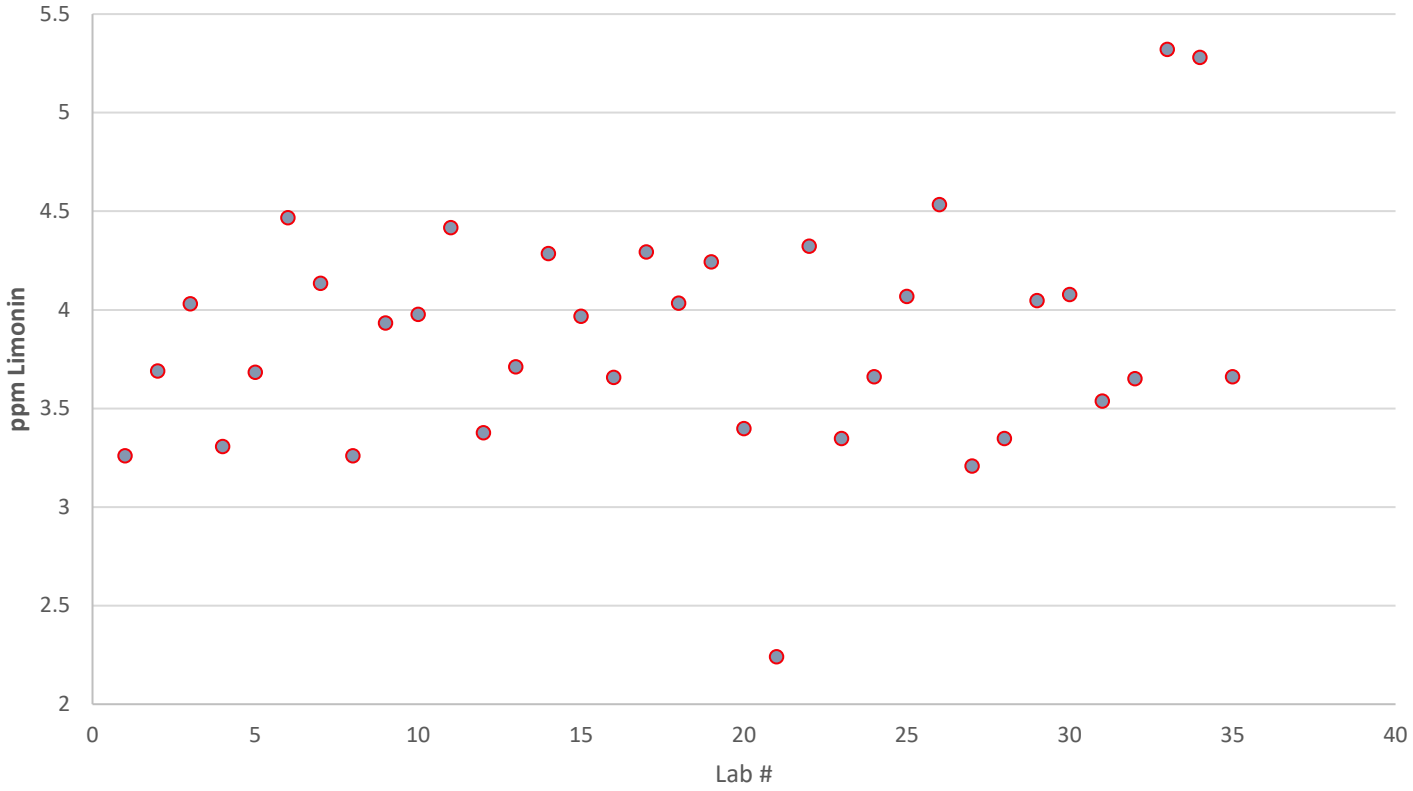
2024 Limonin Ring Test



Average: 3.87 ppm
Min: 2.21 ppm
Max: 5.51 ppm
Std Dev: 0.58

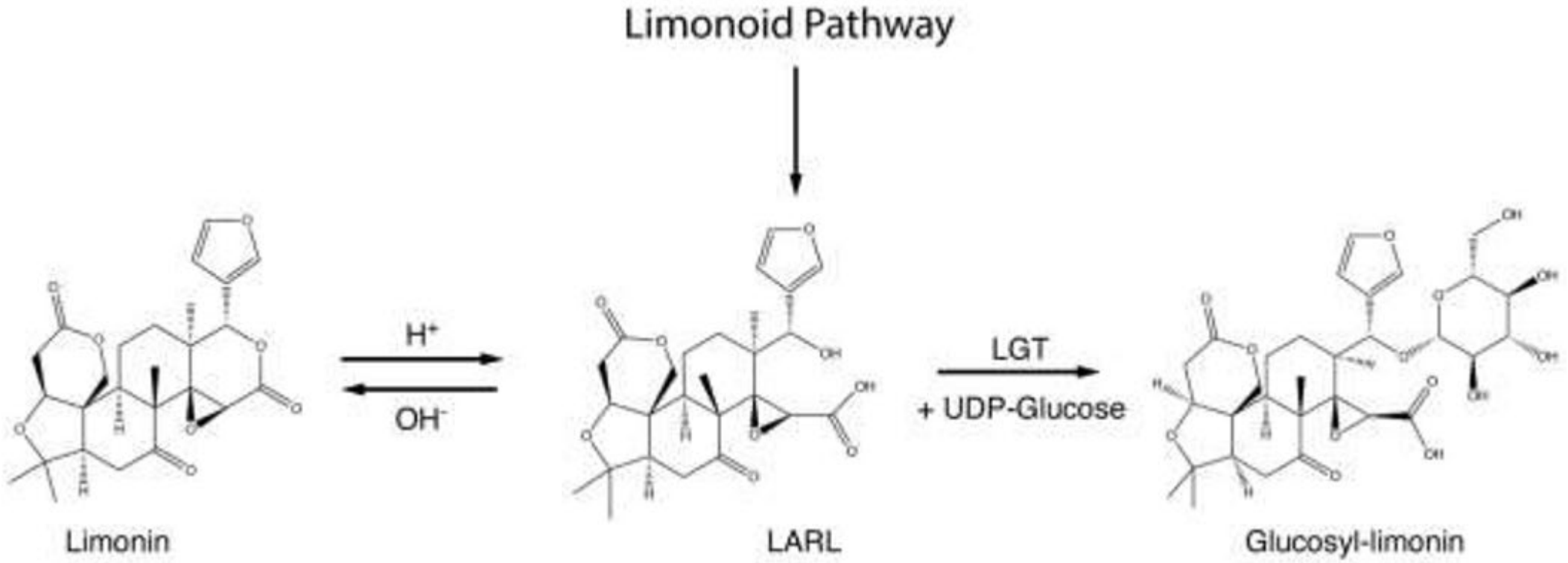
N = 35 Labs

Methodology

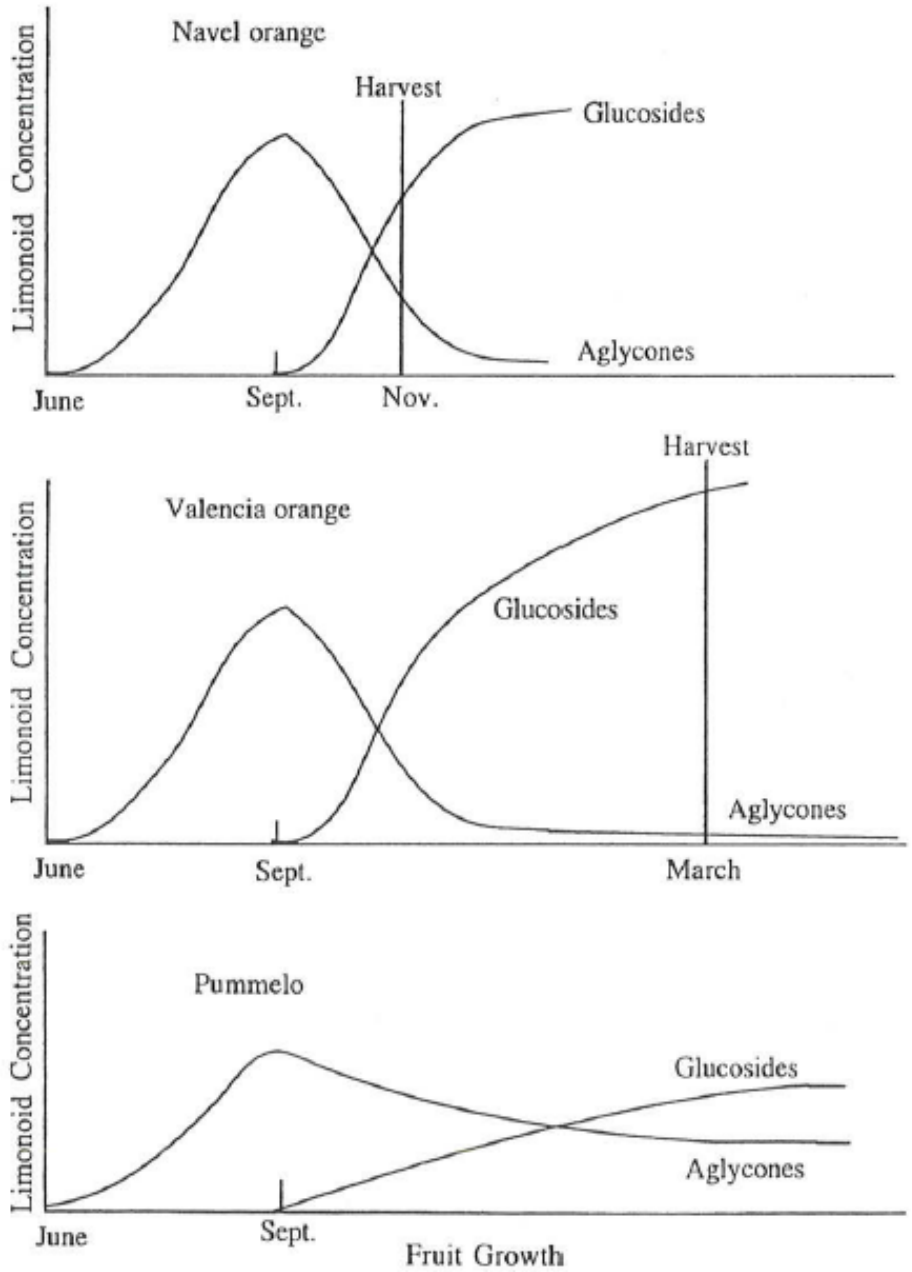
HPLC-UV: 16
LC-MS: 8
LC-MS/MS: 4
Not Reported: 7

Well-characterized, “Mild” OJ used

Measuring Limonin in the Era of Greening



J Sci Food Agric. 2020 October ; 100(13): 4870–4878. doi:10.1002/jsfa.10547.



Flavor Chemistry: 30 Years of Progress, edited by Teranishi *et al.*
Kluwer Academic / Plenum Publishers, New York, 1999.

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The Challenge: What is the “Consumer Relevant” Measurement?

Laboratory Manual

PROCEDURES FOR ANALYSIS OF CITRUS PRODUCTS

Sixth Edition

Manual No. 054R10020.000-6

400 Fairway Avenue, Lakeland, FL 33801 USA

JBT FoodTech

IV. Procedure

1. Heat juice sample of about 60 ml in boiling water bath for 3 – 5 min to develop limonin. Heating is not needed for concentrate and pasteurized juice samples.
2. Centrifuge 25 ml of the juice at 2500 ×g for 10 min
3. Precondition C_{18} cartridges by passing through 2.5 ml of acetonitrile followed by 2.5 ml of HPLC grade water under vacuum until all water just enters the C_{18} bed.
4. Load 2.5 ml of juice supernatant on the preconditioned C_{18} cartridge. For samples with low limonin content, increase load volume accordingly.
5. Slowly filtrate the juice supernatant under vacuum or pressure.

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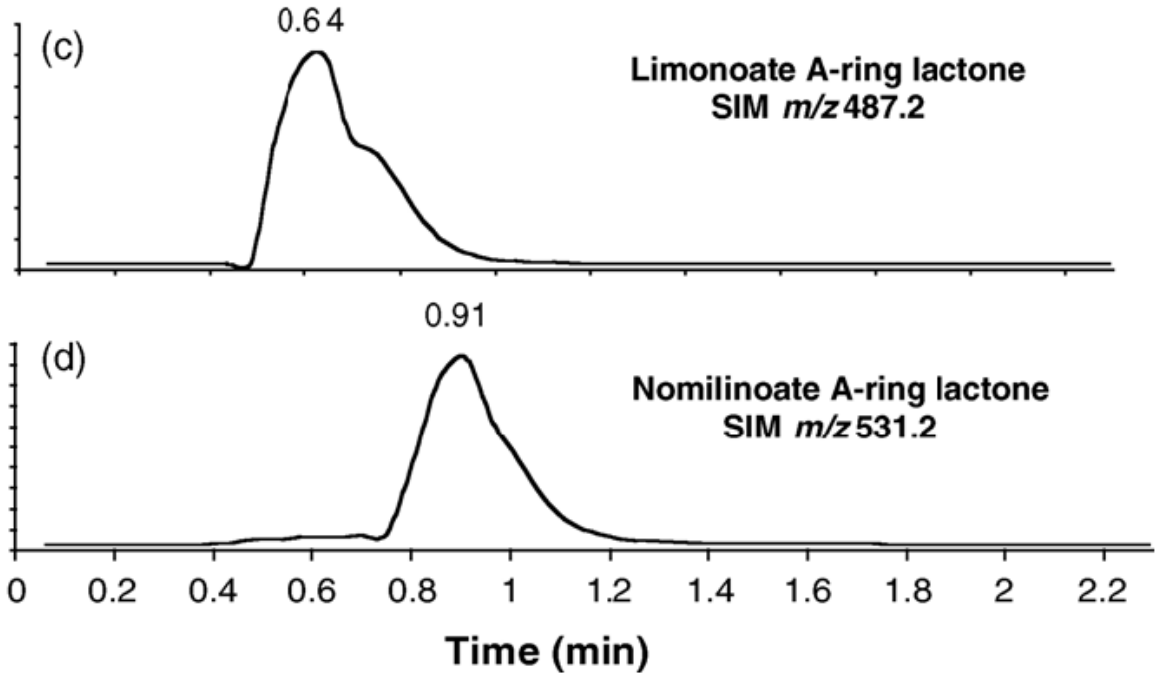
Prior Work (~2005- USDA)



2.3. Preparation of and quantification of LARL and NARL stock solutions

LARL and NARL stock solutions were prepared daily and generated enzymatically utilizing limonoid D-ring lactone hydrolase (LDLH) that was purified as previously described [4]. The reaction mixture consisted of purified LDLH (100 μL), Tris–HCl (120 μL, 1 M, pH 8.0), water (980 μL) and solid limonin or nomilin (2–3 mg). Following incubation at 30 °C (10 h), the reaction mixture was clarified using a centrifuge (14 000 × *g*, 5 min, 4 °C) and applied (1 mL) to a C-18e SPE column (500 mg, Phenomenex, Torrance, CA, USA) prewashed with MeOH (2 mL) and equilibrated in water (2 mL). The flow through and a water wash (2 mL) were discarded. A-ring lactones were eluted with solution A (1.5 mL). LARL and NARL concentrations were established by their

A.P. Breksa III et al. / J. Chromatogr. A 1064 (2005) 187–191

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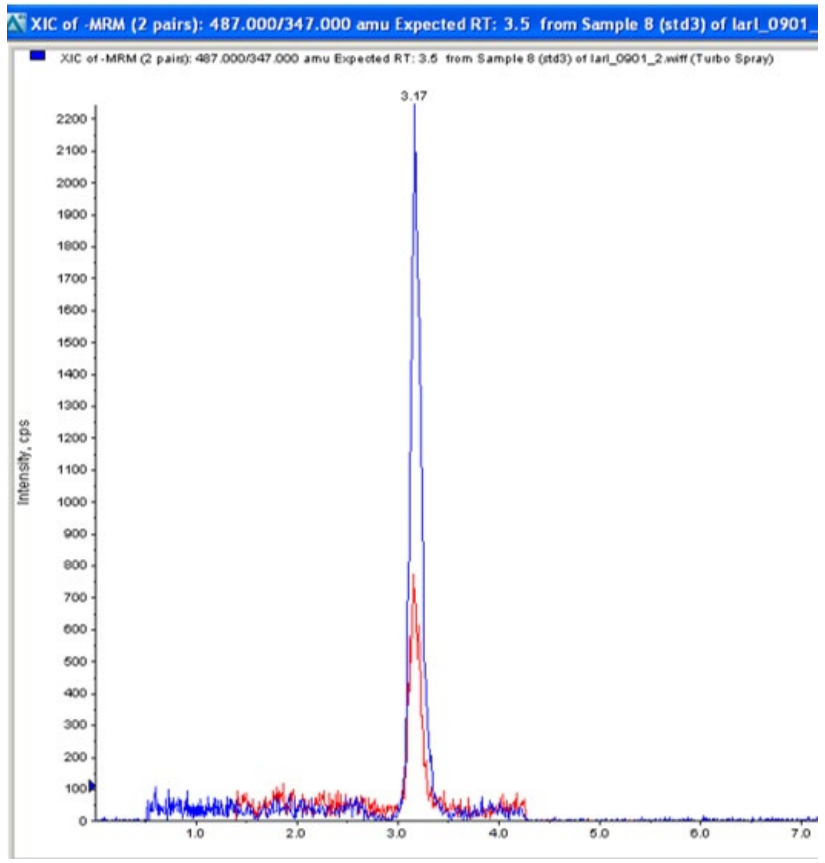
Our Approach

Isolation of LARL via C18 SPE,
Buffer at pH = 6.7



HILIC-MS/MS Method

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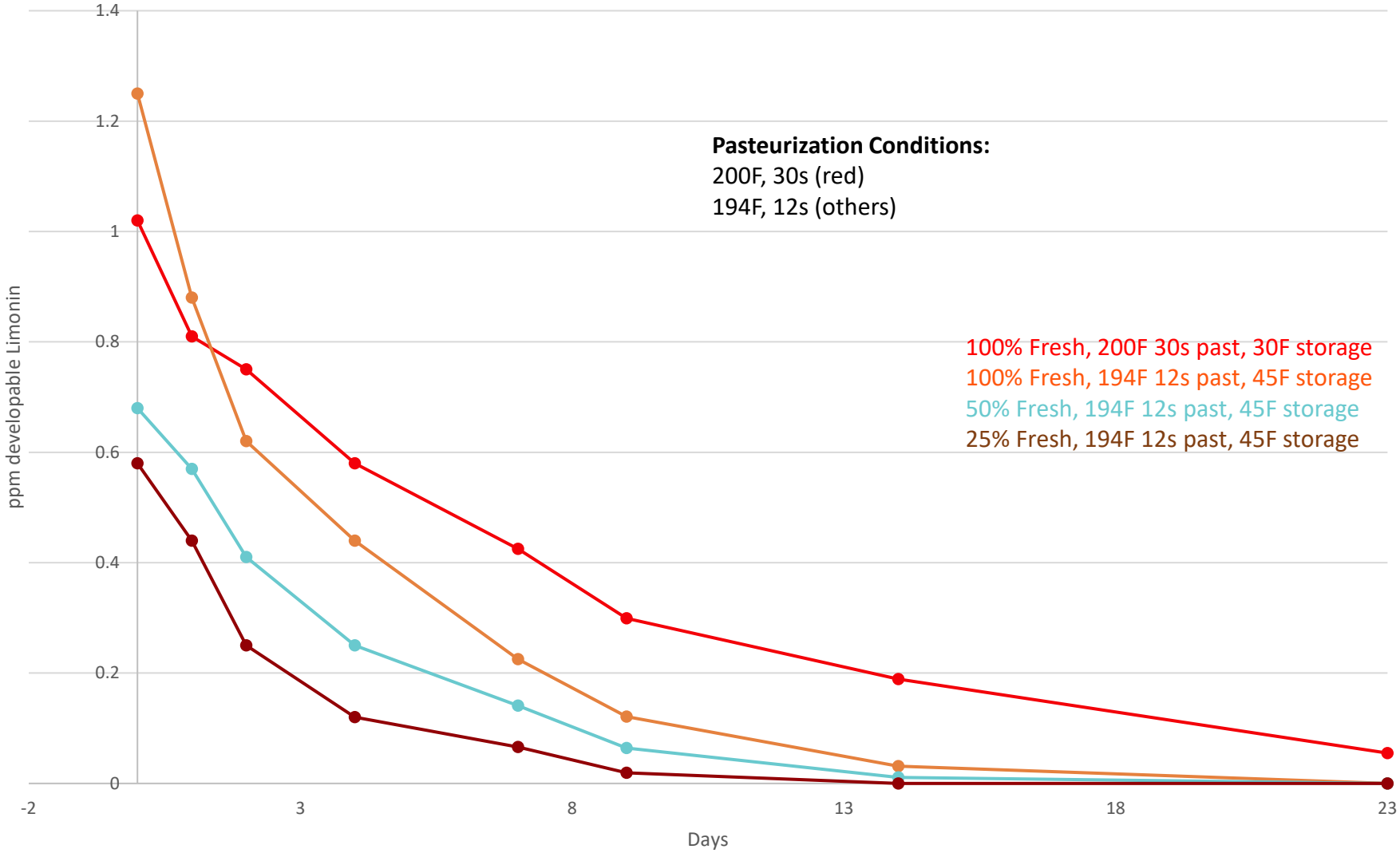
In-House Trials Showed LARL survived pasteurization



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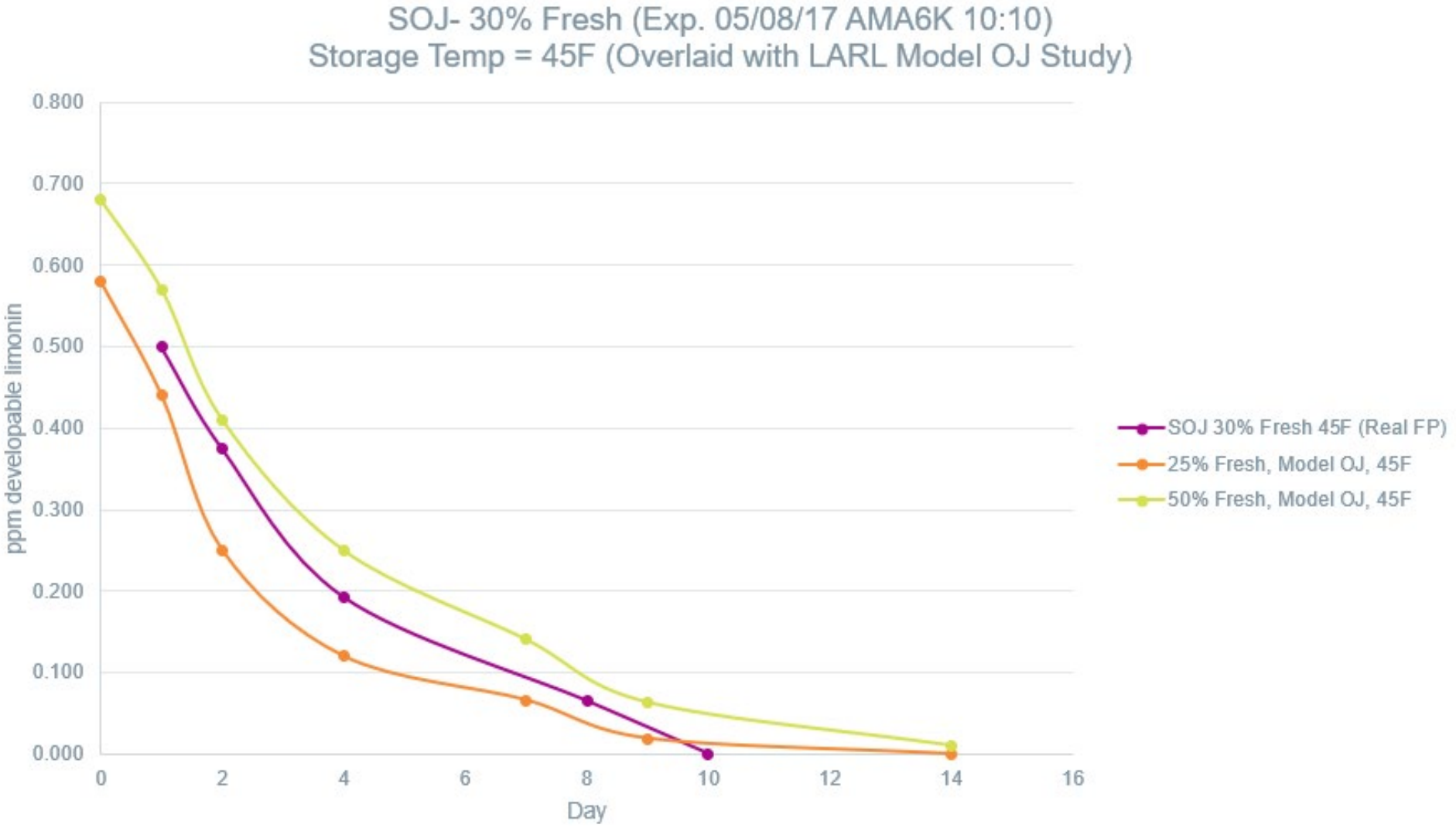
Models Aligned with Real World



Forced Degradation

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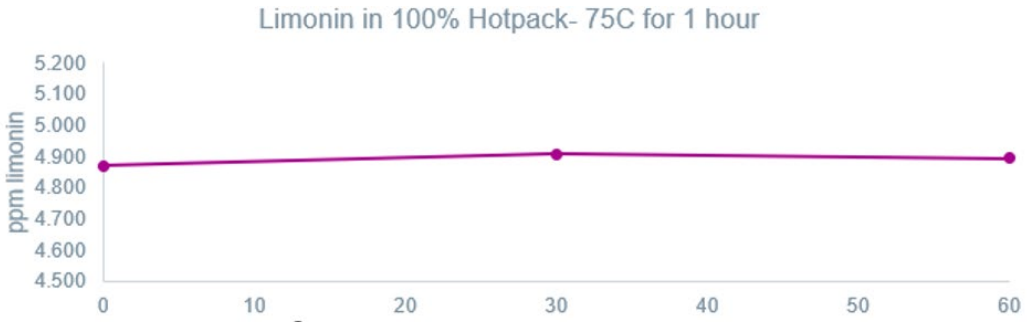
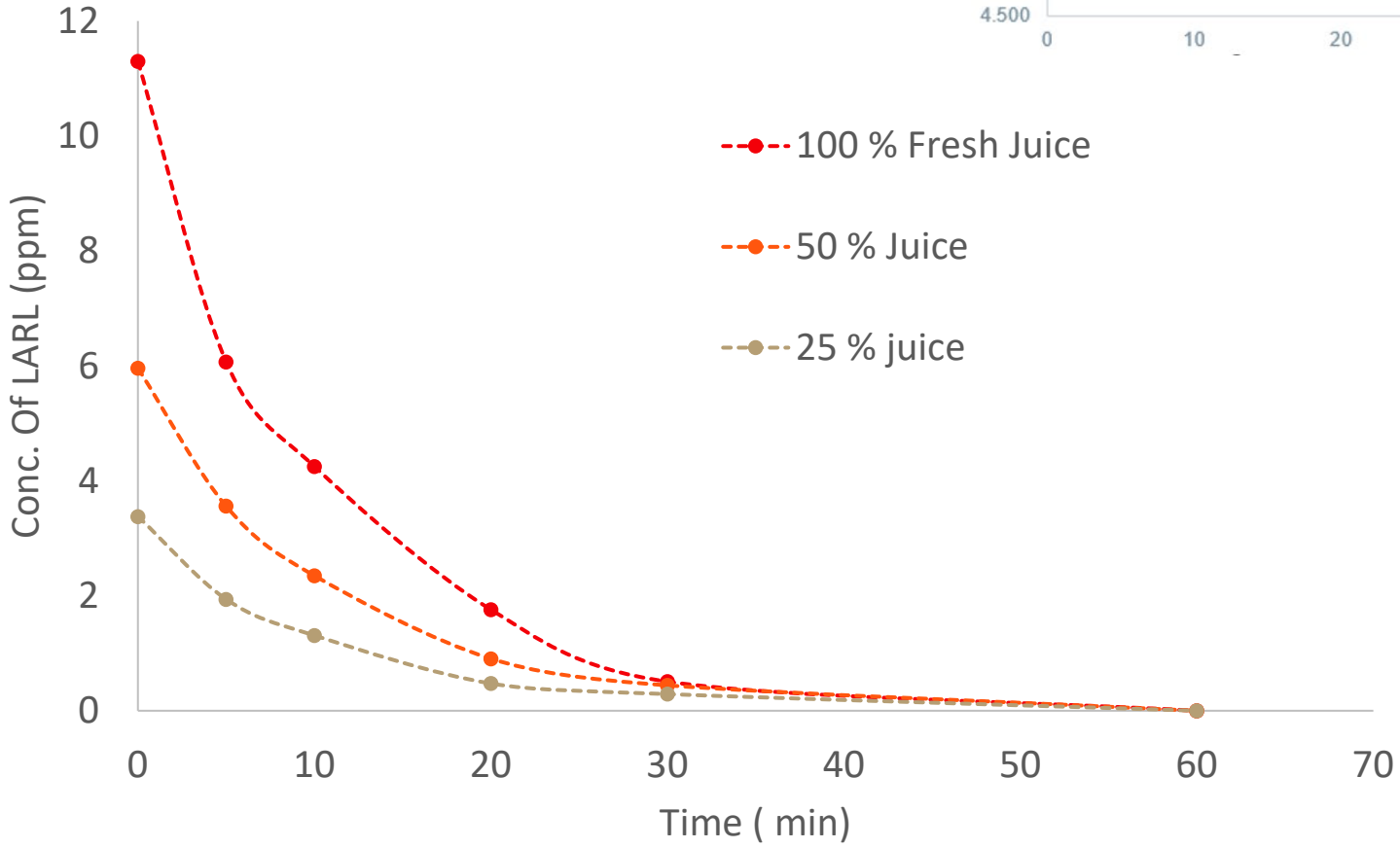
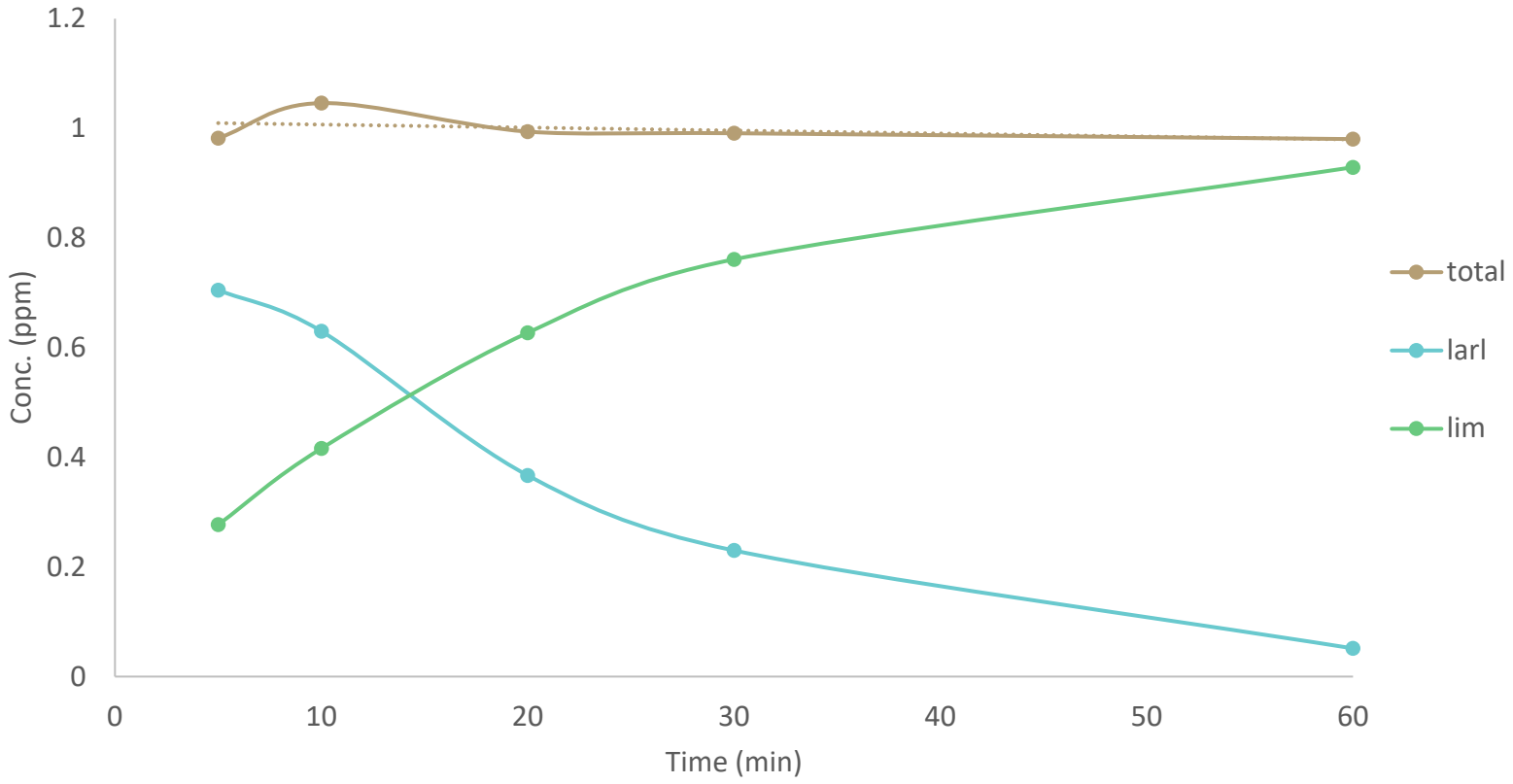


75 C



Mass Balance- 75℃ Limonin vs LARL



- **Fresh Juice needs thermal treatment to fully develop limonin**
  - Recommended protocol: 75°C for 1 hour.
  - Required when juice less than 14 days old irrespective of pasteurization status (LARL survives pasteurization!)
  - Consumer-relevant number IS the fully developed number.
  - **When it doubt, heat.**

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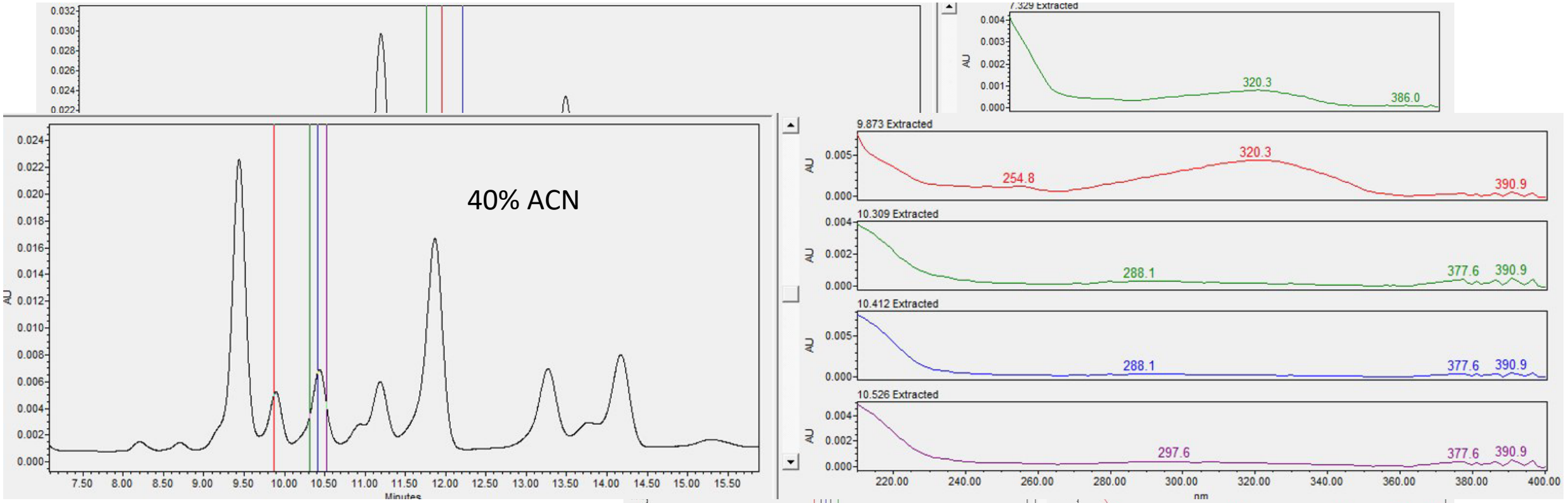
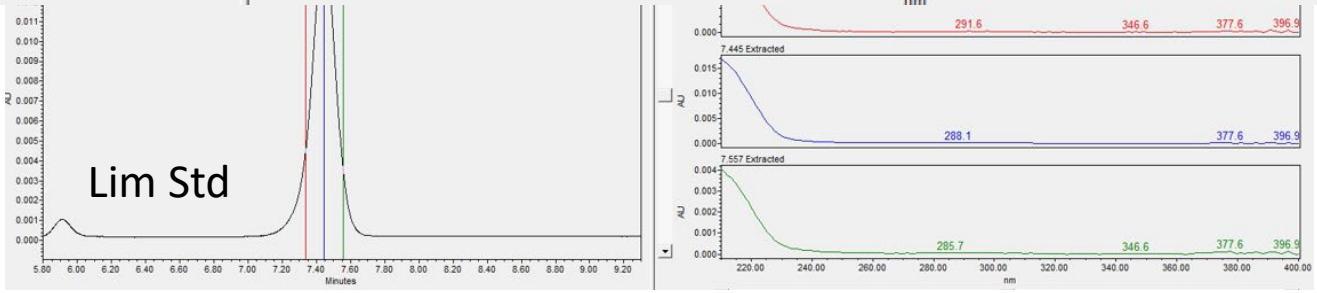
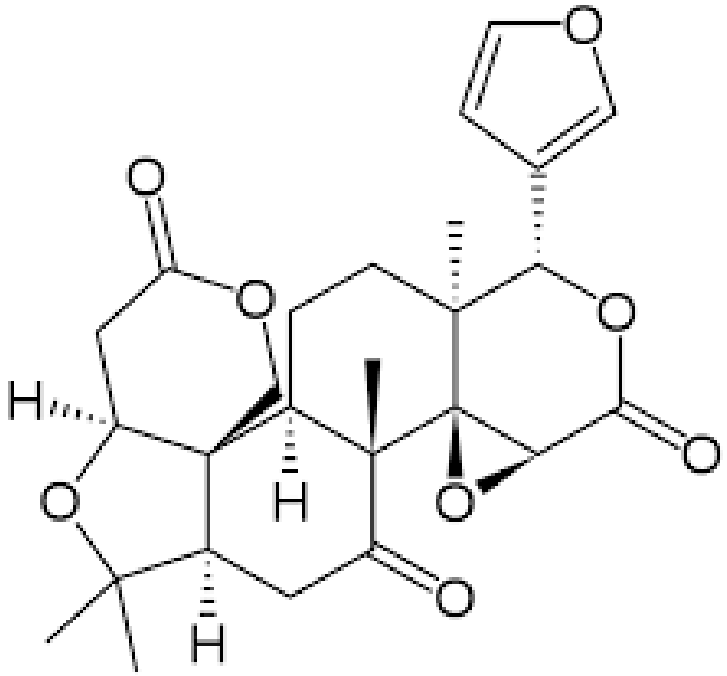
Watchouts!

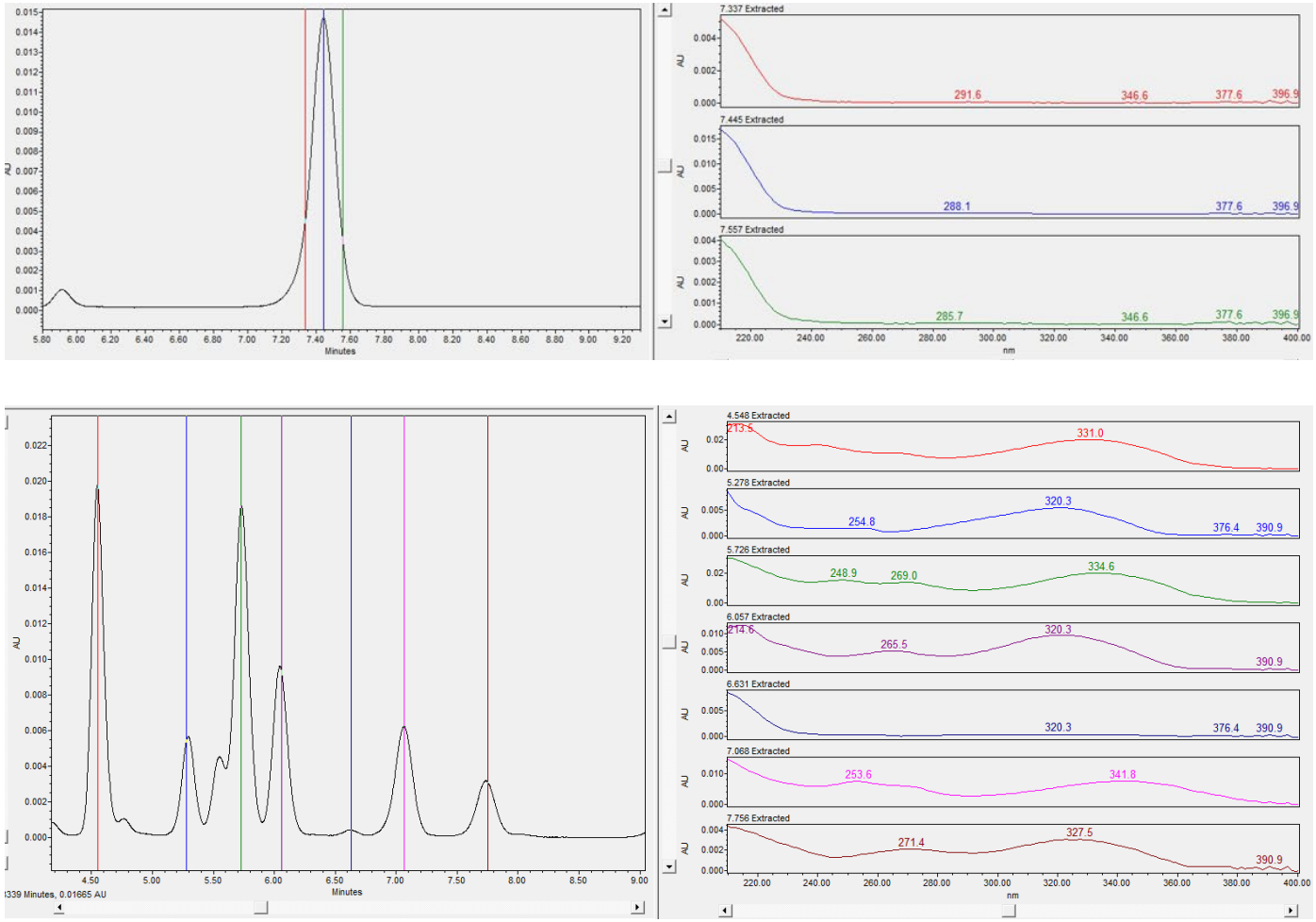




“Mild” vs “Wild”

What can you do?





- Heat if appropriate
- Spectral Purity (if PDA)
- Monitor at ~320nm (if VWD)
- LC-MS if higher volume? (consider sample prep cost)
- QC system

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Limonin QC Program

Sample Qual Example

New QC Rep #	
1	3.56
2	3.46
3	3.45
4	3.42
5	3.42
6	3.44
7	3.49
8	3.59
9	3.49
10	3.56
11	3.50
12	3.51
13	3.58
14	3.52
15	3.54
AVERAGE	3.50
STDEV	0.056
%RSD	1.60
95% CI	0.028

- Original QC created by consensus value (internal + external)
- Re-qualification of new QC from prior QC
- Well-characterized, *typical* juice used.
  - **Should this be reconsidered?**
  - **Typical vs. “Wild” QC? Both?**

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UC DAVIS
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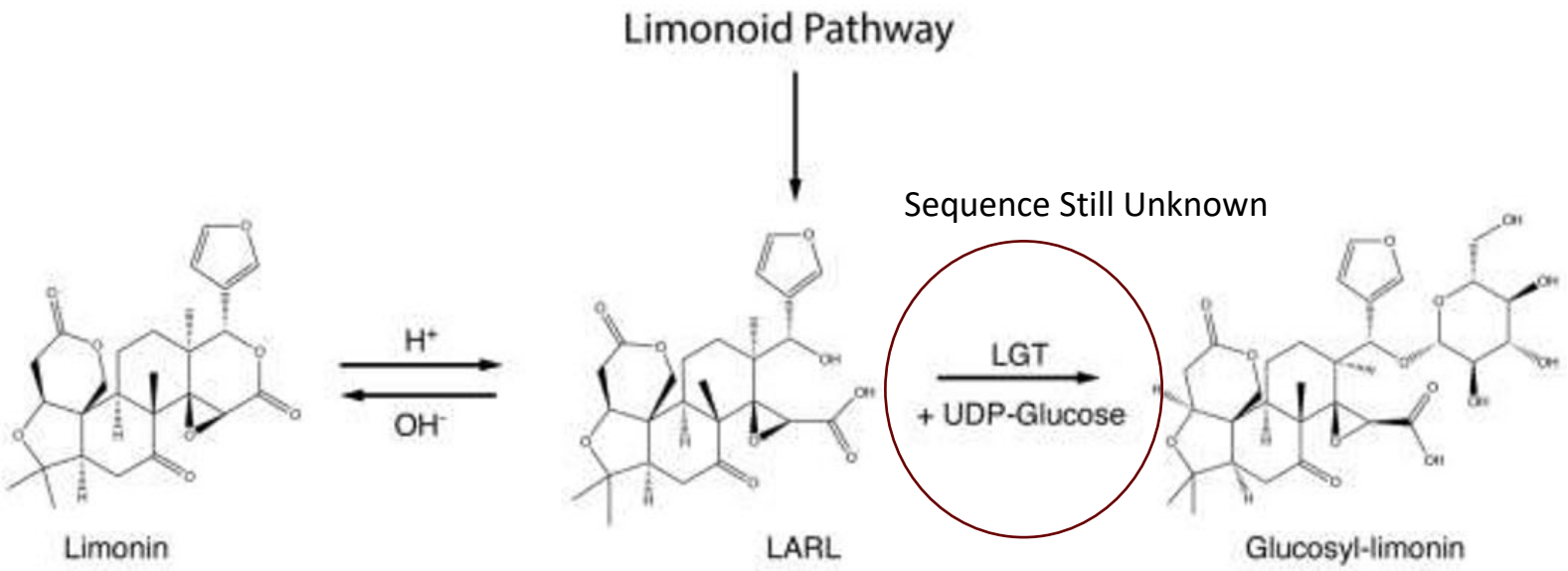

Journal of the Science of Food and Agriculture
SCI where science meets business

Research Article

Functional characterization and reclassification of an enzyme previously proposed to be a limonoid UDP-glucosyltransferase

Youtian Cui, Steven D Allmon, Justin B Siegel

First published: 01 June 2020 | https://doi.org/10.1002/jsfa.10547 | Citations: 6

Abstract

BACKGROUND

A major problem in the orange industry is 'delayed' bitterness, which is caused by limonin, a bitter compound developing from its non-bitter precursor limonoate A-ring lactone (LARL) during and after extraction of orange juice. The glucosidation of LARL by limonoid UDP-glucosyltransferase (LGT) to form non-bitter glycosyl-limonin during orange maturation has been demonstrated as a natural way to debitter by preventing the formation of limonin.

RESULT

Here, the debittering potential of heterogeneously expressed glucosyltransferase, maltose-binding protein (MBP) fused to *cu*GT from *Citrus unishiu Marc* (MBP-*cu*GT), which was previously regarded as LGT, was evaluated. A liquid chromatography – mass spectrometry (LC–MS) method was established to determine the concentration of limonin and its derivatives. The protocols to obtain its potential substrates, LARL and limonoate (limonin with both A and D ring open), were also developed. Surprisingly, MBP-*cu*GT did not exhibit any detectable effect on limonin degradation when Navel orange juice was used as the substrate; MBP-*cu*GT was unable to biotransform either LARL or limonoate as purified substrates. However, it was found that MBP-*cu*GT displayed a broad activity spectrum towards flavonoids, confirming that the enzyme produced was active under the conditions evaluated *in vitro*.

CONCLUSION

Our results based on LC–MS demonstrated that *cu*GT functionality was incorrectly identified. Its active substrates, including various flavonoids but not limonoids, highlight the need for further efforts to identify the enzyme responsible for LGT activity to develop biotechnology-based approaches for producing orange juice from varietals that traditionally have a delayed bitterness.

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Benchtop Fruit Processing

5 LB Samples

Fruit Firmness

Hand Extraction

Water Bath Pasteurization

Fruit Attributes

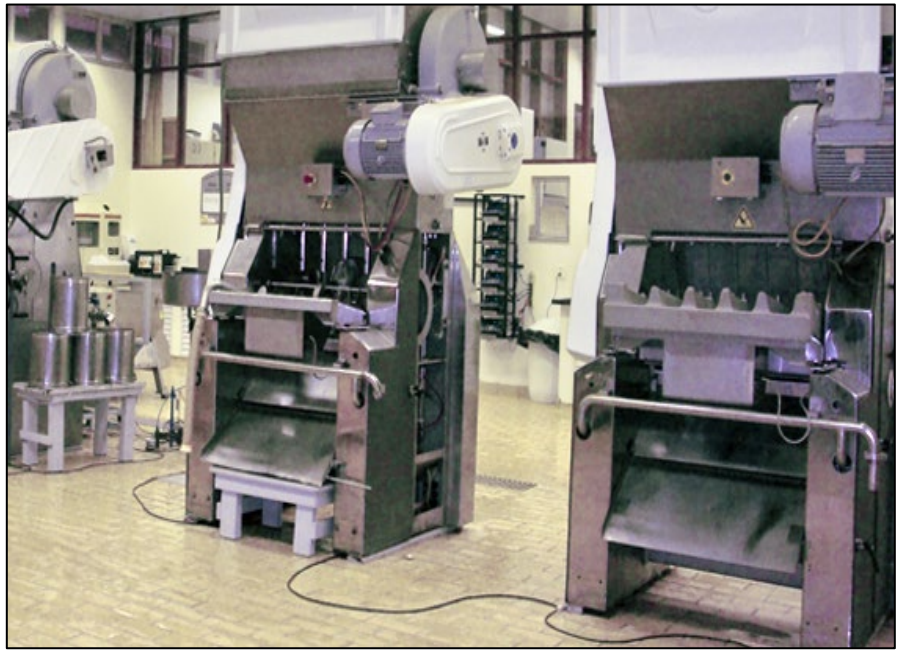
Size, Firmness, Juice yield

Juice Attributes

Brix, Acid, Ratio, Limonin, Color, Flavor

Benchtop Limonin vs. Commercial Limonin

Hand Extraction: 0.9 ppm



Pilot Scale: 2.0 ppm

Comparison with Valencia fruit in 2023.

Standardization is Critical!


We tested 1000s of samples representing 100s of genetically unique mandarin hybrids.

Juice Quality Mandarin-types All Locations

Juice Quality

Several mandarins were low limonin & high Brix!

Limonin (Bitter)

20
18
16
14
12
10
8
6
4
2
0

Brix (Sweet)

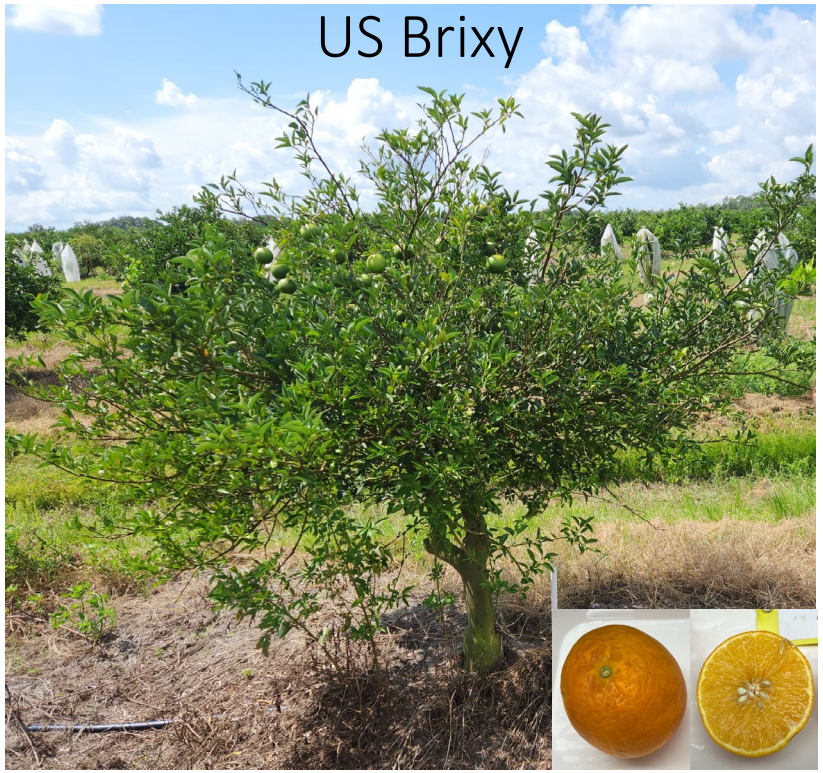
6
7
8
9
10
11
12
13
14
15

Optimal Range

Sun Dragon
Sugar Belle
Released Mandarin-types
Unreleased Mandarin-types

Top 3 Mandarins

- Good tree health (87th percentile)
- Fruit size similar to Sweet Orange
- 12.1 Brix (95th percentile)
- 2.0 ppm Limonin (74th percentile)
- High potential for juicing



- Average tree health (48th percentile)
- Fruit size larger than Sweet Orange
- 11.5 Brix (93rd percentile)
- 1.2 ppm Limonin (90th percentile)
- High potential for juicing

- Good tree health (98th percentile)
- Fruit size similar to Murcott
- 12.8 Brix (98th percentile)
- 4.0 ppm Limonin (42nd percentile)
- High potential easy-peel, low-seed table fruit

*Based on 2 seasons of data from 8-12 topwork trees of each variety. Block located in South Florida.

- 1 acre each of the top 3 mandarins planted August 2025

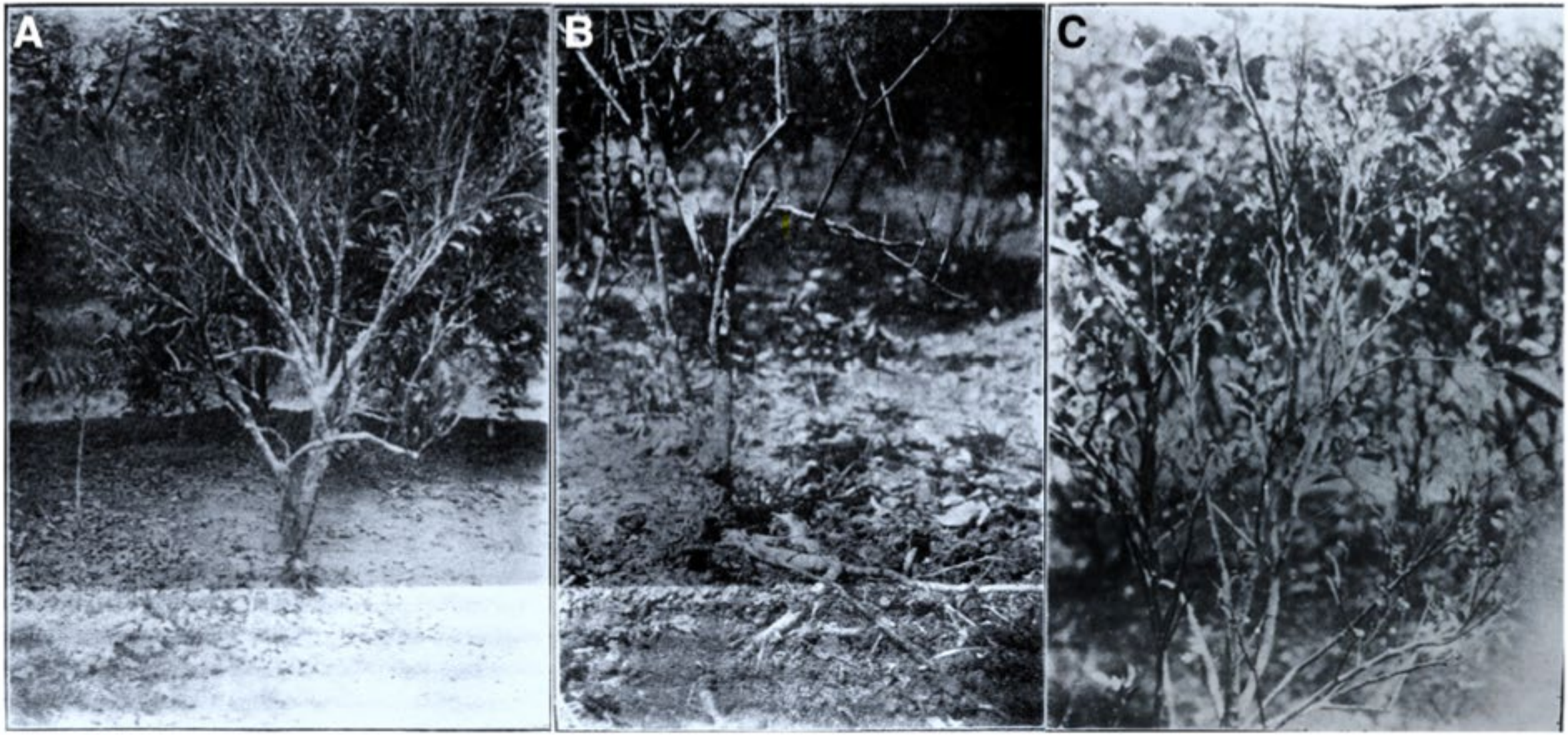


FIGURE 3

Three photographs of citrus huanglongbing published by Tu (1932). **A,** Sweet orange tree (*Citrus sinensis* Osbeck) dying from yellowing disease; **B,** root rot of a sweet orange tree affected by yellowing disease; and **C,** untimely flowering in sweet orange affected by mottled leaf disease.

Prof. C. Tu (涂治) of Plant Pathology in Lingnan University performed a citrus disease survey in fall 1930 (Tu 1932). He raised a concern about the future of citriculture in Guangdong because of a dreadful malady of "yellowing" disease. He also reported a mottled leaf disease as a different disease described by Reinking (1919). The yellowing disease was described as such: "The trees so affected become sickly looking and much stunted. Usually the leaves have a pale, yellowish cast. At first the twigs gradually die back. In the more advanced stage, there is considerable defoliation especially during the dry seasons. Finally, the tree dies at the age of its greatest fruitfulness" (Tu 1932). Two photographs of yellowing disease in sweet orange were presented, one tree severely defoliated and the other with rotted roots (Fig. 3A and B). "Yellowing was linked to poor development in root systems. The fibrous roots were usually sloughed off. The main roots became rotten and got broken, and the lateral roots were much blackened." This was probably the first record description of HLB as a root rot disease. Mottled leaf was listed as a problem after yellowing. Sweet orange (*C. sinensis*) was more susceptible than mandarin (*C. reticulate*). Diseased leaves showed characteristic mottling (Fig. 3C but not clear). "The disease usually spread from the top downwards. In the advanced stage, the twigs might die back. Sometimes, multiple buds were formed. Later, considerable defoliation occurred." Prof. Tu mentioned two additional symptoms, smaller leaves and untimely flowers (Fig. 3C).

TABLE 1
Records of citrus export in the unit of Dan (approximately 60 kg) in four cities in Guangdong Province between 1925 and 1934 (Jiang et al. 1935b)

Year	Shantou	Guangzhou	Jiangmen	Gongbei
1925	193,935	11,959	11,326	3,063
1926	192,200	11,792	15,010	1,814
1927	224,341	8,975	33,276	5,196
1928	248,333	8,604	36,601	15,351
1929	270,482	10,020	15,319	24,987
1930	235,469	17,468	18,452	17,430
1931	220,786	24,215	43,675	6,294
1932	109,450	9,911	61,111	7,001
1933	126,518	10,894	84,898	5,830
1934	60,379	5,039	52,488	3,829

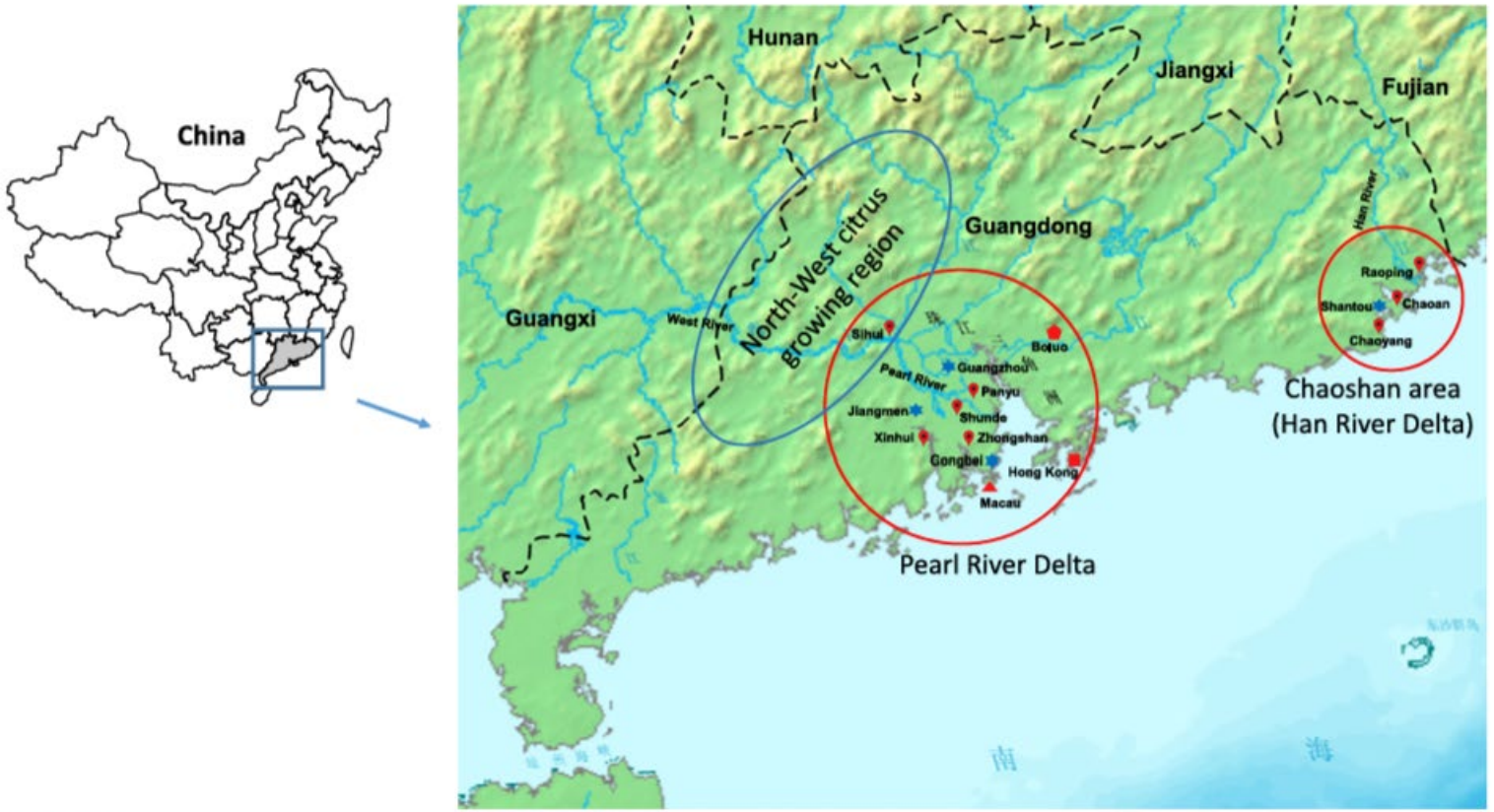


FIGURE 2
Topology map of Guangdong and neighboring provinces in southern China. Three major citrus production areas at present and in the past are circled. Red circles indicate major citrus planting regions before the 1990s. The blue circle is the major citrus production region since the mid-1990s.

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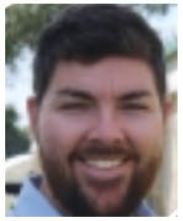

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